International Journal of Agricultural
Science and Research (IJASR)
ISSN (P): 2250-0057; ISSN (E): 2321-0087
Vol. 7, Issue 4, Aug 2017, 269-276

OVER-EXPRESSION OF *ARABIDOPSIS PHYTOCHELATIN SYNTHASE* (*ATPCS1*) GENE IN *ESCHERICHIA COLI* CONFERS ENHANCED TOLERANCE TO CADMIUM

SRINIVAS NAIK KETHAVATH[1], SWATHI BAI MOODAVATH[2], HANUMALAL NUNAVATH[3] & BUKYA BHIMA[4]

[1]*Centre for Plant Molecular Biology, Osmania University, Hyderabad, Telangana, India*

[2, 3, 4]*Department of Microbiology, Osmania University, Hyderabad, Telangana, India*

ABSTRACT

Phytochelatin synthesis (PCS), the key enzyme involved in heavy metal detoxification and accumulation has been studied extensively for the purpose of Phyto remediation. In this study, we have isolated the AtPCS1 gene from Arabidopsis thaliana, which is considered as a potentially accumulator of heavy metals. The 1458bp CDNA of AtPCS1 was isolated, cloned and sequenced. The deduced amino acid sequence of the C - terminal region of the AtPCS1 contained homologous regions of functional domains of metallothionein and thioredoxin. Heterologous expression of AtPCS1 gene in E. coli resulted in an enhanced accumulation and tolerance to heavy metal Cadmium. This enzyme has the potential to develop transgenic plants with enhanced tolerance to heavy metals for phytoremediation.

KEYWORDS: Bioremediation, Phytochelatins & Phytochelatin Synthase

Received: Jun 15, 2017;**Accepted:** Jul 11, 2017; **Published:** Jul 18, 2017; **Paper Id.:** IJASRAUG201734

Original Article

INTRODUCTION

Environmental contamination with heavy metals, is implicated in many plant and animal diseases and causes crop losses. This universal problem, poses a challenge with a price tag for remediation estimated at upward of $200 billion, in the US alone (Abhilash et al., 2009). In bioremediation, microbes and plants are used as ecological remediators, to reclaim the clean environment at a limited cost. Toxic heavy metals can be eliminated from the soil, by specialized microbes and plants, termed as hyper accumulators, which are capable of accumulating and tolerating the toxic levels of the heavy metals in their shoots (Cunningham et al., 1995; Salt et al., 1998). However, this ability of bioremediation is limited to few organisms. A promising way of improving the bioremediation potential would be to genetically engineer the organisms with enhanced abilities to tolerate and accumulate toxic heavy metals and metalloids. Though many efforts have been made to engineer the microbes and plants for an enhanced metal accumulation potential, but limited success was achieved in this direction (Brunetti et al., 2011; Liu et al., 2012; Wang et al., 2012).

The heavy metal cadmium (Cd) and metalloid arsenic (As) are among the most toxic carcinogenic elements, which show high affinity towards thiols (Tripathi et al., 2013).Cadmium is a widespread heavy metal that is mainly released into the environment by anthropogenic activities and it is highly toxic to both plants and animals. Cadmium decreases the growth rate of plants by affecting various aspects of metabolism, mainly in normal uptake and utilization of macro and micronutrients (Metwally et al., 2005). Similarly, two common forms of arsenic in the environment are the oxyanions, arsenate (As V) and arsenite (As III). As V interferes with phosphate metabolism, while As III has a high affinity towards sulphydryl groups and binds to proteins affecting their structures and catalytic

functions.

Plants have developed various defence mechanisms for protection against various environmental stimuli. Exposure of plants to heavy metals, such as cadmium, arsenic, mercury or lead, promotes the synthesis of plant metal chelators, viz., metallothioneins (MTs) and phytochelatins (PCs) (Cobbett et al., 2002). MTs are small Cys- rich proteins encoded by multigene families; while, PCs are enzymatic ally synthesized Cys- rich polypeptides that are likely to be ubiquitous in plants, and possibly present in animals also (Lee et al., 2002). The thiol group of Cys has been reported to play an important role in both the homeostasis of essential heavy metal ions and the sequestration of various non-essential toxic metal ions at the sub cellular level (Rauser, 1995).

PCs are a family of cysteine-rich, thiol-reactive peptides that bind many toxic metals and metalloids, making them good candidates for genetically enhanced phytoremediation strategies (Cobbett and Meagher, 2002). PCs were first identified in the yeast *Schizosaccharomycespombe* and subsequently have been found in plants, fungal species, marine diatoms and certain animals (Cobbett, 1999). PCs are non-translationally synthesized small polypeptides and are synthesized by the transpeptidation of γ-glutamylcysteinyl dipeptides from glutathione (GSH) with the help of Phytochelatin Synthase (PCS). PCS is a constitutively expressed enzyme and it is known for its post-translational activation in the presence of heavy metals. PCS genes have been identified in many organisms such as *Arabidopsis* (*AtPCS1*), wheat (*TaPCS1*), *S. pombe*(*SpPCS1*) and *Caenorhabditis elegans*(*CePCS1*)(Clemens et al., 2001; Sanita di Toppi et al., 2002).

The genes coding for PCS have been cloned from a variety of organisms and have been over-expressed in bacteria, *Arabidopsis, Nicotianaglauca*and rice (Lee et al., 2003; Li et al., 2004). Over-expression of *AtPCS1* in *Arabidopsis*, Mustard and tobacco had resulted in enhanced tolerance towards As and Cd (Lee et al., 2003; Li et al., 2004; Brunetti et al., 2011; Gasic and Korban, 2007; Wojas et al., 2010a & 2010b). Also, transgenic plants expressing different PCS genes, viz., *NnPCS1*, *TcPCS1, PtPCS1* and *TaPCS1*in *Arabidopsis*, tobacco and poplar resulted in accumulation of various metalloids and heavy metals, viz., Cd, As, Pb and Zn (Liu et al., 2012; Couselo et al., 2010; Adams et al., 2011).

In this paper, we report the effects of overt expression of Arabidopsis AtPCS1 genes in *E. Cali*. We investigated whether the over-expression is capable of increasing Cd and As tolerance in the recombinant E coli.

MATERIALS AND METHODS

Atpcs1 Isolation and Cloning

Total RNA, from 15-day-old *Arabidopsis* seedlings (ecotype Columbia) was isolated using the RNeasy plant mini kit (Qiagen) following the manufacturer's instructions. Two micro grams of total RNA were reverse transcribed using the Super Script™ III First-Strand Synthesis System (Thermo Fisher Scientific). Two micro litres of c DNA was used to perform PCR using *pfu* polymerase employing forward and reverse primers, viz., 5′-GGATCCATGGCTATGGCGAGTTTA-3′ and 5′-GAGCTCCTAATAGGCAGGAGCAGCGAG-3′, specific to *AtPCS1* coding sequence. The underlined regions in the primers depict *Bam*HI and *Sac*I restriction sites introduced for subsequent cloning purposes. Amplified fragments of 1470 bp were cloned at *some* site of p Blue script KS (+) (Stratagene, USA), then transformed into *E. coli* Top10 cells and was sequenced using automated DNA sequence.

Development of Recombinant E. Coli Expressing Atpcs1 and Protein Accumulation

The CDNA coding sequence of *AtPCS1* from pBSSK (+) was excised by *Bam*H I and *Eco*R I and was sub-cloned downstream to T7 promoter in frame with 6X His-tag of pET-21a (+) expression vector, which was introduced into *E. coli* Rosetta™ (DE3) pLysS cells. The recombinant clones were confirmed through restriction analysis. *E. coli* cells, harbouring *AtPCS1* were grown in LB medium containing 50µg/ml of kanamycin at 37°C and 200 RPM until the cell density reached O.D 0.6–0.8. Isopropyl β-D-1-thiogalactopyranoside (IPTG) was added to the final concentration of 500 µM and cultures were grown at 30°C. After 4–6 h of incubation, cells were harvested by centrifugation at 8000 × g for 15 min at 4°C. The cells were lysed and the expressed proteins analyzed according to Kamarthapu et al. (2008).

Growth Kinetics of E. Coli Under Cd Stress

In order to study the response of recombinant bacteria expressing *AtPCS1* to 100µM of Cd stress, growth of transforming *E. coli* Rosetta™ (DE3) pLysS cells with pET-28a (+) (empty vector), recombinant plasmid pET-28a (+)-AtPCS1 and mock *E. coli* Rosetta™ (DE3) pLysS cells was examined in LB medium with and without Cd treatment. Primary culture of these was allowed to grow at 37°C for overnight. Overnight grown culture (100µl) was diluted into 2.4 ml of LB broth, of which 750µl was inoculated into 20 ml of secondary culture and allowed to grow at 37 °C for approximately 3h until O.D.600 reached up to 0.4. In secondary culture, a final concentration of 0.1mm of IPTG was added for induction at 28°C. After a gap of 1 h, CD was added to a final concentration of 100µM. Thereafter, absorbance was measured at 600 nm wavelength at each interval of 1h.

Estimation of CD Accumulation in E. Coli

For quantitative determination of cadmium, *E. coli* cells grown for 5 h in the presence of IPTG and Cd (100µM) were harvested, rinsed three times using LB medium and dried at 60°C for 2 days. Dried cells were digested in HNO_3 (70 %)/$HClO_4$ (30 %) and mineralization was carried out in a microwave oven as described by Dwivedi et al. (2010). The level of metal was quantified using inductively coupled plasma mass spectrometer (Agilent 7500 cx).

RESULTS

Cloning and Bacterial Expression of Atpcs1

An approximate 1.5Kb cDNA coding region of *AtPCS1* was amplified employing total RNA isolated from *Arabidopsis* seedlings. The isolated *AtPCS1* gene was ligated into *Sma*I-digested pBSSK (+) vector, then transformed into *E. coli* Top10 cells and sequenced. The isolated cDNA nucleotide sequence revealed an identity of 100% with that of *AtPCS1* and *Leucaena leucocephala* phytochelatin synthase (*LlPCS1*). Later, *AtPCS1*gene was sub-cloned downstream to T7 promoter of pET-21a (+) expression vector, which was introduced into *E. coli* Rosetta™ (DE3) pLysS host cells. Restriction analysis of recombinant pET-21a (+) clones carrying *AtPCS1* gene disclosed the presence of ~5.5 Kb and ~ 1.5 Kb bands corresponding to vector and *AtPCS1* gene, respectively. *E. coli* Rosetta™ (DE3) pLysS cells harbouring *AtPCS1* gene, when induced with 1 mM IPTG at 37°C and 225 rpm for 4 h, showed a~56 kDa in the induced samples and was absent in the control samples (Fig.1)

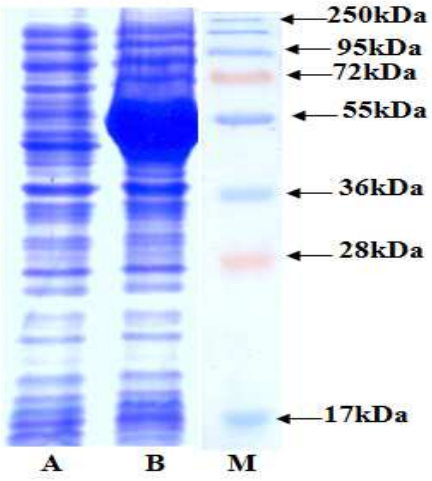

Figure 1: SDS-PAGE (12%) Gel Analysis of AtPCS1 Protein Expression in *E. Coli* Rosetta™ (DE3) pLysS Cells

Lane A: Cell lysates from *E. coli* cells containing empty vector pET-21a (+).

Lane B: Cell lysates from *E. coli* cells containing recombinant plasmid pET-21a (+) with AtPCS1.

Lane M: protein marker. *E. coli* cells were grown until OD 600 reached to 0.4 followed by addition of IPTG for induction of expression of AtPCS1 protein.

Expression of Atpcs1 Confers CD Tolerance in E.Coli

Growth inhibition was observed in Cd-treated control *E. coli* cells (Rosetta™ (DE3) pLysS) as well as transformed with pET-21a (+) empty vector. However, in the absence of Cd, *E. coli*cells transformed with pET-21a (+) grew normally. In contrast, the growth of the Cd treated *E. coli* cells expressing AtPCS1 protein increased when compared to control cells (Fig. 2A).

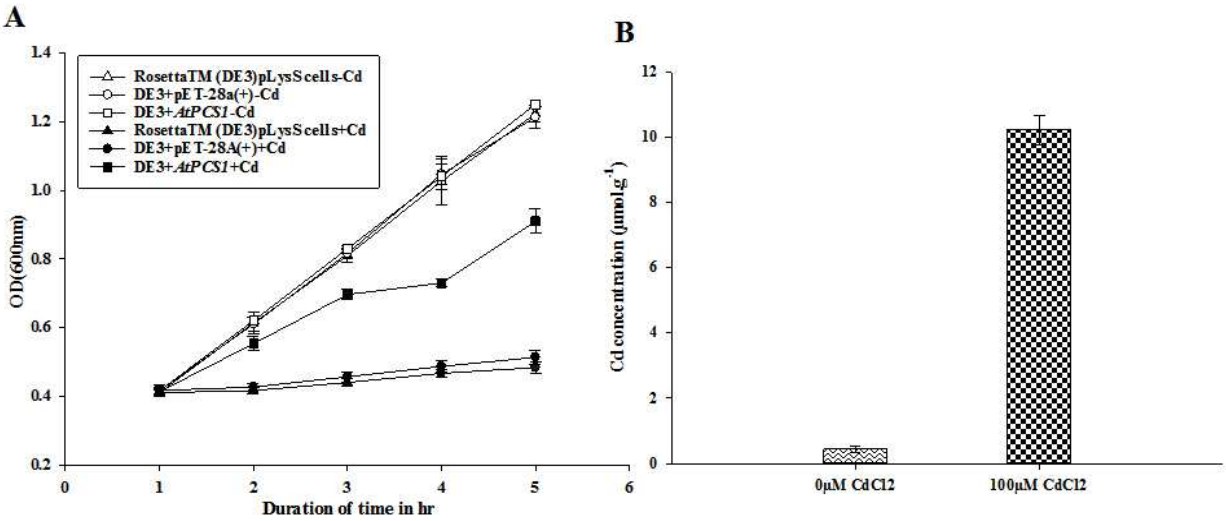

Figure 2: Growth and Metal Accumulation of *E. coli* Under Cd Stress

Effect of Cd on growth of *E. coli* cells (mock; triangle), empty vector pET-21a (+) (circle) and recombinant plasmid pET-21a (+) with AtPCS1 (rectangle), respectively Empty and filled markers represent with and without Cd

treatment. The mean of three independent experiments are plotted with error bars indicating ±SD.

B) Estimation of intracellular cadmium concentration of *E.coli* cells expressing *AtPCS1*, in the absence and presence of Cd (100 µM) after IPTG induction

To study the tolerance to Cd stress, *in vitro* total PC and Cd content was measured in *E. coli* cells expressing AtPCS1 protein in the presence and absence of 100µM Cd. Similarly, growing bacteria expressing AtPCS1 on Cd-enriched medium led to increased intra cellular Cd content. A mean of 0.44 ±0.03 and 10.23±0.45µmolg^{-1}, intracellular Cd was accumulated in *E. coli* cells grown in the presence and absence of Cd, respectively (Fig. 2B). Furthermore, marked increase in the As intracellular metal content was also observed when bacteria were grown on As-enriched medium. In contrast, no significant intracellular accumulation of these metals could be detected when *E. coli* without *Atpcs1* gene were grown on metal-enriched media.

DISCUSSIONS

Phytochelatins, a class of post translationally synthesized peptides, play a pivotal role in heavy metal, primarily Cd, tolerance in plants and fungi by chelating these substances and decreasing their free concentrations. PCS is constitutively expressed, but its activity is dependent on the presence of a heavy metal (Vatamaniuk et al., 2004). This investigation deals with cloning of *Arabidopsis phytochelatin*gene (*AtPCS1*) and its effects on tolerance to Cd in *E. coli*

The 1458 bp cDNA sequence of *AtPCS1* was isolated. The sequence and deduced amino acid sequence of AtPCS1 revealed 95-100% homology with that of other previously reported *Arabidopsis* PCS and showed 80-90% homology with *Brassica* species thus indicating the highly conserved nature of the enzyme. Amino acid sequence of C-terminal region of the AtPCS1 contained homologous regions to functional domains of metallothionein and thioredoxin. Therefore, it was suggested that C-terminal region of the PCS play an important role as regulatory region for PC synthesis. The arrangements of Cys residues, –Cys–Cys–Arg–Glu–Thr–Cys–Val–Lys–Cys–, is reminiscent of those found in metallothionein and thioredoxin (Cobbett et al., 2002; Capitani et al., 2000; Romero-Isart et al., 2002). The sequence homology identified pfam05023 domain which is specific to Phytochelatin synthase. This domain is responsible for the synthesis of heavy-metal-binding peptides (phytochelatins) from glutathione and related thiols. The crystal structure of a member of this family shows it to possess a papain fold. The enzyme catalyses deglycination of a GSH donor molecule. The enzyme contains a catalytic triad of cysteine, histidine and aspartate residues. Another domain DUF 1984 (Domain of unknown function) was also found in the members of this family of functionally uncharacterized domains are found at the C-terminus of plant phytochelatin synthases.

Upon IPTG induction *E. coli* Rosetta (DE3) pLysS cells expressing AtPCS1 revealed a characteristic band corresponding to 56.3kDa band of the expected size, whereas the uninduced cells failed to exhibit this band (Fig.1). *E. coli* Rosetta (DE3) pLysS cells expressing *AtPCS1* led to significant tolerance when grown in the presence of Cd (Fig. 2A) possibly by the synthesis of PCs by PCS activity. The expression of *AtPCS1* enhanced cellular Cd content ranged between 23% and 35% in recombinant *E.coli* cells. Earlier, it was reported that growing bacteria and yeast expressing *At PCS* on Cd-enriched medium led to a 20-fold increase in the intracellular Cd, Cu and as content. (Sauge-Merle et al., 2003; Singh et al, 2008) Similarly, *E.coli* cells expressing PCS gene from *Anabaena* sp. PCC 7120 provided tolerance against Cd, heat and salt stress (Chaurasia et al., 2008). The enhanced tolerance to various stresses as well as heavy metal is attributed to strong nucleophilic sulfhydryl groups of the cysteine present in PCs' reaction with a broad spectrum of agents ranging

from free radicals, reactive oxygen species to heavy metals (Rabenstein, 1989) and detoxifying them, resulting into tolerance (Chaurasia et al., 2008; Gill and Tuteja, 2010).

CONCLUSIONS

This investigation deals with the cloning and expression of *Arabidopsis phytochelatin synthase* gene (*AtPCS1*) in *E. coli*. The recombinant *E. coli* showed both enhanced tolerance and higher accumulation of Cd compared to the respective control.

ACKNOWLEDGEMENTS

The authors are grateful to the University Grants Commission (UGC), New Delhi, Govt. of India for financial support under DRS and UPE-FAR program.

REFERENCES

1. *Abhilash, P.C., Jamil, S., Singh, N. (2009).Transgenic plants for enhanced biodegradation and phytoremediation of organic xenobiotics. Biotechnol. Adv. 27, 474-488.*
2. *Adams, J.P., Adeli, A., Hsu, C.Y., Harkess, R.L., Page, G.P., dePamphilis, C.W., Schultz, E.B., Yuceer, C. (2011) Poplar maintains zinc homeostasis with heavy metal genes HMA4 and PCS1. J. Exp. Bot. 62, 3737-3752.*
3. *Brunetti, P., Zanella, L., Proia, A., De Paolis, A., Falasca, G., Altamura, M.M., Sanita di Toppi, L., Costantino, P., Cardarelli, M. (2011). Cadmium tolerance and phytochelatin content of Arabidopsis seedlings over-expressing the phytochelati synthase gene AtPCS1. J. Exp Bot. 62, 5509-5519*
4. *Capitani, G., Markovic-Housley, Z., DelVal, C., Morris, M., Jansonius, J.N., Schurmann (2000).Crystal structures of two functionally different thioredoxins in spinach chloroplasts, J. Mol. Biol. 302,135-154*
5. *Chaurasia,N., Mishra, Y., Rai, L.C.(2008) Cloning, expression and analysis of phytochelatin synthase (pcs) gene from Anabaena sp. PCC 7120 offering multiple stress tolerance in Escherichia coli, Biochem.Biophys. Res. Commun. 376, 225-230.*
6. *Clemens, S., Schroeder, J.I., Degenkolb, T.(2001) Caenorhabditis elegans expresses a functional phytochelatin synthase. Eur. J.Biochem. 268, 3640-3643.*
7. *Cobbett, C., Goldsbrough, P.(2002) Phytochelatins and metallothioneins: roles in heavy metal detoxification and homeostasis, Plant Biol. 53, 159-182.*
8. *Cobbett, C.S.(1999) A family of phytochelatin synthase genes from plant, fungal and animal species. Trends Plant Sci. 4, 335-337.*
9. *Cobbett, C.S., Meagher, R.B.(2002) Arabidopsis and the Genetic Potential for the Phytoremediation of Toxic Elemental and Organic Pollutants. The Arabidopsis Book / American Society of Plant Biologists 1, 32*
10. *Couselo, J.L., Navarro-Avino, J., Ballester, A (2010). Expression of the Phytochelatin Synthase TaPCS1 in Transgenic Aspen, Insight into the Problems and Qualities in Phytoremediation of Pb. Int. J. Phytoremediation 12, 358-370.*
11. *Cunningham, S.D., Berti, W.R., Huang, J.W.W. (1995) Phytoremediation of contaminated soils Trends Biotechnol 13, 393-397*
12. *Dwivedi, S., Tripathi, R.D., Srivastava, S., Singh, R., Kumar, A., Tripathi, P., Dave, R., Rai, U.N., Chakrabarty, D., Trivedi, P.K., Tuli, R., Adhikari,B., Bag, M.K. (2010). Arsenic affects mineral nutrients in grains of various Indian rice (Oryzasativa L.) genotypes grown on arsenic-contaminated soils of West Bengal. Protoplasma. 245,113-124.*
13. *Gasic, K., Korban, S.S.(2007). Transgenic Indian mustard (Brassica juncea) plants expressing an Arabidopsisphytochelatin*

synthase (AtPCS1) exhibit enhanced as and Cd tolerance. Plant Mol. Biol. 64, 361-369.

14. *Gill, S.S., Tuteja, N. (2010) Reactive oxygen species and antioxidant machinery in abiotic stress tolerance in crop plants Plant Physiol. Biochem. 48, 909-930.*
15. *Kamarthapu, V., Rao, K.V., Srinivas, P.N., Reddy, G.B., Reddy, V.D. (2008). Structural and kinetic properties of Bacillus subtilis S-adenosylmethioninesynthetase expressed in Escherichia coli.Biochimicaet.BiophysicaActa. 1784, 1949-58.*
16. *Lee, S., Korban, S. (2002) Transcriptional regulation of Arabidopsis thaliana phytochelatin synthase (AtPCS1) by cadmium during early stages of plant developmen. Planta. 215: 689-693.*
17. *Lee, S., Moon,J.S., Ko, T.S.,Petros, D., Goldsbrough, P.B.,Korban,S. (2003).Overexpression of Arabidopsisphytochelatin synthase paradoxically leads to hypersensitivity to cadmium stress. Plant Physiol. 131, 656-663.*
18. *Li, Y., Dhankher, O.M., Carreira, L., Lee, D., Chen, A., Schroeder, J.I., Balish, R.S, Meagher, R.B. (2004). Overexpression of phytochelatin synthase in Arabidopsis leads to enhanced arsenic tolerance and cadmium hypersensitivity. Plant Cell Physiol. 45, 1787-1797.*
19. *Liu, G.Y., Zhang, Y.X., Chai, T.Y. (2011). Phytochelatin synthase of Thlaspicaerulescens enhanced tolerance and accumulation of heavy metals when expressed in yeast and tobacco. Plant Cell Rep. 30, 1067-1076.*
20. *Liu, Z., Gu, C., Chen, F., Yang, D., Wu, K., Chen, S., Jiang, J., Zhang, Z. (2012). Heterologous expression of a Nelumbonuciferaphytochelatin synthase gene enhances cadmium tolerance in Arabidopsisthaliana.ApplBiochemBiotechnol. 166,722-734.*
21. *Metwally, A., Safronova, V.I., Belimov, A.A., Dietz, K.J. (2005). Genotypic variation of the response to cadmium toxicity in Pisumsativum L. J. Exp. Bot. 56, 167-178.*
22. *Rabenstein, D. L. (1989). Metal complexes of glutathione and their biological significance, pp. 147–186.InD. Dolphin, O. Avra-movic, and R. Poulson [eds.], Glutathione: Chemical, biochem-ical, and medical aspects. Wiley.*
23. *Rauser, W.E. (1995). Phytochelatins and Related Peptides' Structure, Biosynthesis, and Function Plant Physiol.109, 1141-1149.*
24. *Romero-Isart, N., Vasak, M. (2002) Advances in the structure and chemistry of metallothioneins . In org Biochem 88, 388-396*
25. *Salt, D.E., Smith, R.D., Raskin, I. (1998). Phytoremediation. Annu. Rev. Plant Physiol. Plant Mol. Biol. 49, 643-668.*
26. *Sanita di Toppi, L., Prasad, M.N.V., Ottonello, S. (2002). Metal chelating peptides and proteins in plants. In: Prasad MNV, Strzaka K (eds) Physiology and biochemistry of heavy metal detoxification and tolerance in plants. Kluwer, Dordrecht, pp 59-93.*
27. *Sauge-Merle, S., Cuine, S., Carrier, P., Lecomte-Pradines, C., Luu, D.T., Peltier, G. (2003). Enhanced toxic metal accumulation in engineered bacterial cells expressing Arabidopsisthalianaphytochelatin synthase. Appl. Environ. Microbiol. 69, 490-494.*
28. *Singh, S., Lee, W., DaSilva, N.A., Mulchandani, A., Chen, W. (2008). Enhanced arsenic accumulation and removal by engineered yeast cells expressing Arabidopsisthalianaphytochelatin synthase. Biotechnol. Bioeng. 99, 333-340.*
29. *Tripathi, P., Tripathi, R.D., Singh, R.P., Dwivedi, S., Chakrabarty, D., Trivedi, P.K., Adhikari, B. (2013). Arsenite tolerance in rice (Oryzasativa L.) involves coordinated role of metabolic pathways of thiols and amino acids. Environ. Sci. Pollut. Res. Int. 20, 884-896.*
30. *Vatamaniuk, O. K. (2004). Phytochelatin synthase, a dipeptidyltransferase that undergoes multisite acylation with c-*

glutamylcysteine during catalysis. J. Biol. Chem. 279, 22449-22460.

31. *Wang, F., Wang, Z., Zhu, C. (2012). Hetero expression of the wheat phytochelatin synthase gene (TaPCS1) in rice enhances cadmium sensitivity. Acta. Biochim. Biophys. Sin (Shanghai). 44, 886-893.*
32. *Wojas, S., Clemens, S., Skłodowska, A., Maria Antosiewicz, D. (2010a). Arsenic response of AtPCS1- and CePCS-expressing plants-effects of external As(V) concentration on As-accumulation pattern and NPT metabolism. J Plant Physiol. 15, 169-175.*
33. *Wojas, S., Ruszczyńska, A., Bulska, E., Clemens, S., Antosiewicz, D.M. (2010b). The role of subcellular distribution of cadmium and phytochelatins in the generation of distinct phenotypes of AtPCS1- and CePCS3-expressing tobacco. J Plant Physiol. 167, 981-988.*